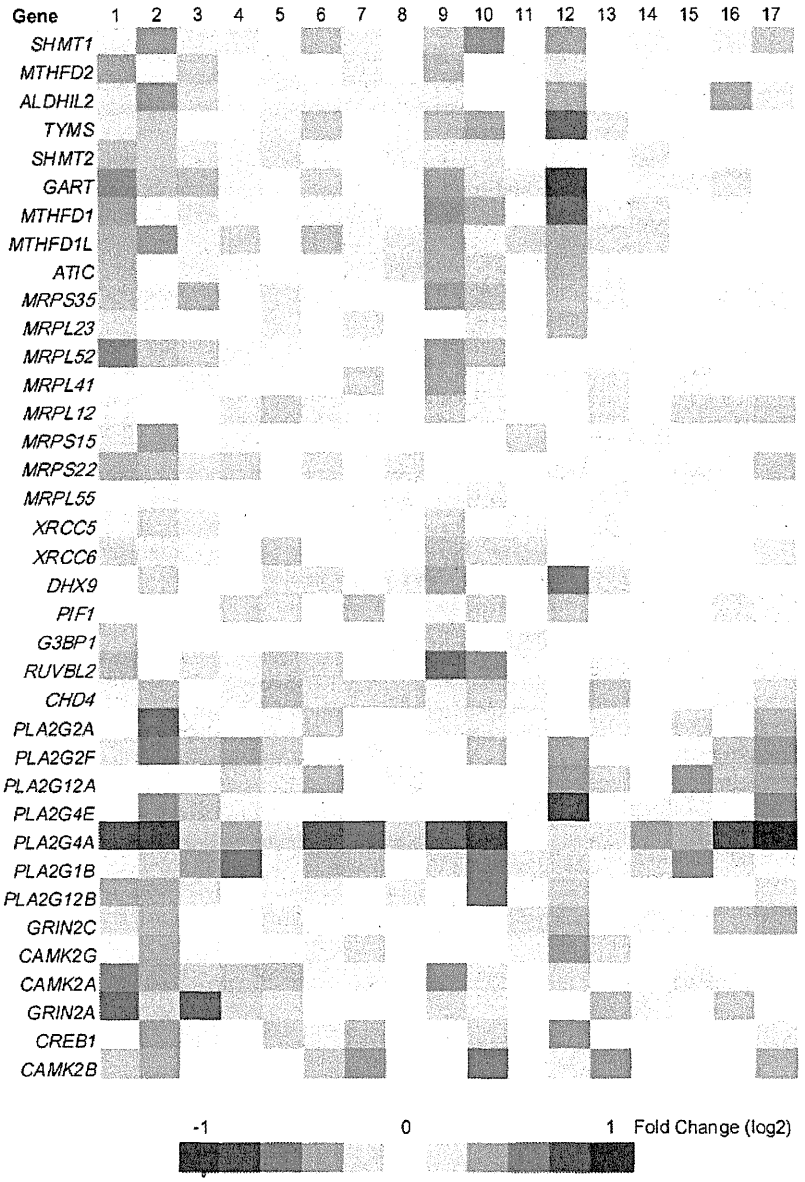

Figure 3. Heat map of the 37 genes with core enrichment from up-regulated genes sets. GSEA analysis was carried out to identify if any pre-defined gene set showing different expression levels between the group of higher IQ twins and the group of their lower IQ co-twins. Gene set databases BioCarta, KEGG, Reactome, and Gene Ontology were applied separative. Gene sets meeting the cutoff FDR *q*-value of 0.25 were subjected to leading edge analysis to determine the genes with core enrichment. The result was a list of 37 genes and we generated a heat map accordingly. Red and blue cells signify genes that were either up- or down-regulated, respectively, after the expression levels of the twins with higher IQ scores compared to their co-twins. The scale represents fold changes in log2 values, according to the color map at the bottom of the figure. The general tendency of higher expression levels in twins with higher IQ scores from the gene *SHMT1* to *CHD4*, and their lower expression levels from the gene *PLA2G2A* to *CAMK2B*, was visualized.
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mitoribosome function might play a role in the maintenance of neuronal biological processes.

Apart from mitochondrial ribosomal protein-related gene sets, "ATP-dependent DNA Helicase Activity" from the GO database was also found. DNA helicases are molecular motor proteins that use nucleoside 5′-triphosphate hydrolysis as a source of energy to open energetically stable duplex DNA into single strands. As such, they are essential in almost all aspects of cellular DNA machinery including DNA replication, repair, recombination, and transcription [31]. Of which, *XRCC5* and *XRCC6* encode the two subunits of the Ku protein, which plays an important role in the repair of double-stranded DNA breaks and telomere protection [32]. In neurodegenerative diseases, such as Alzheimer's disease, where cellular damage due to oxidative stress is proposed to contribute to pathophysiology, reduced Ku protein expression and its DNA binding activity have been thought to be involved [33]. Of the remaining five helicases denoted, *G3BP1* was demonstrated to play an essential part in proper embryonic growth and neonatal

Table 3. Summary of the most commonly shared gene sets showing the same tendency (up-regulated in the higher IQ twin or in the lower IQ twin) according to the pair-wise GSEA test.

Up-regulated in	Gene sets database	Gene set	Shared by twin pair ID
Higher IQ twins	BioCarta	MCM pathway	1,9,12,17
	KEGG	Proteasome	1,3,9,10,17
		Oxidative phosphorylation	1,9,10,12,16
		Ribosome	1,6,9,10,12,16
		DNA replication	1,3,8,9,10,12,17
		Valine leucine and isoleucine degradation	1,3,10,12,17
		Mismatch repair	1,3,9,12,17
		Peroxisome	1,9,10,12,17
	Reactome	RNA polymerase I promoter opening	1,3,9,11,12,13,16,17
		Electrotransport chain	1,3,9,10,12,13,16,17
		Packaging telomere ends	1,3,9,11,12,16,17
		Telomere maintenance	1,3,9,11,12,16,17
	Gene Ontology	Structural constituent of ribosome	9,10,12,17
		Microbody	1,9,12,17
		Peroxisome	1,9,12,17
		S-phase of mitotic cell cycle	1,8,15,17
		Small conjugating protein specific protease activity	1,9,15,17
Lower IQ twins	BioCarta	n/a	n/a
	KEGG	Neuroactive ligand receptor interaction	1,3,16,17
		Taste transduction	1,3,9,13,17
	Reactome	Cell-cell adhesion systems	1,3,9,17
	Gene Ontology	Anion transport	1,5,16,17
		Cation channel activity	1,9,16,17
		Cell-cell signaling	1,9,16,17
		Collagen	1,9,16,17
		Extracellular matrix part	1,9,16,17
		Extracellular region part	1,9,16,17
		G-protein coupled receptor protein signaling pathway	1,9,16,17
		Gated channel activity	1,9,16,17
		Intercellular junction	6,9,16,17
		Metal ion transmembrane transporter activity	1,9,16,17
		Second messenger mediated signal	1,9,16,17

n/a indicates no gene set was shared by at least 4 twin pairs.

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survival [34]. Although the direct associations between these seven genes and cognitive abilities have not been depicted, mutations in a list of DNA repair-related genes have already been reported to cause mental retardation [8]. As such, one hypothesis we proposed here is that the up-regulated expression of these helicases might provide better protection from oxidative damages and, thus, improve neuronal function and survival, which could bring forth higher levels of intelligence (or in other words, less compromised) as a phenotype.

On the other hand, the pair-wise GSEA, along with leading edge analysis, identified *CHRNA2* which encodes the α2 subunit of nicotinic acetylcholine receptors (nAChRs). Initially related to nicotine dependence, the role of nAChRs in cognitive performance has gained attention because nicotine is considered a powerful enhancer of cognitive capabilities [35] via the interaction of nicotine and nAChRs [36].

Additionally, two potassium voltage-gated channel-coding genes, *KCNE2* and *KCNQ3*, were identified. Voltage-gated ion channels possess diverse functions, include regulating neurotransmitter release, heart rate, insulin secretion, neuronal excitability, epithelial electrolyte transport, and smooth muscle contraction. By assembling with KCNQ2 or KCNQ5, KCNQ3 forms the M channel, a slow activating and deactivating potassium channel that plays a critical role in the regulation of neuronal excitability [37]. In addition to being identified as one cause for a dominantly inherited form of human generalized epilepsy, called benign familial neonatal convulsions, the electrogenic characteristics of KCNQ/M channels have importance in controlling intrinsic firing patterns of principal hippocampal neurons, thus, further modulating hippocampal learning and memory [38]. Of note, rats treated with Linopirdine, an M channel-specific inhibitor,

Table 4. Up-regulated genes with core enrichment shared by co-twins of multiple pairs.

Up-regulated in	Gene Sets database	Gene	Shared by twin pair ID
Higher IQ twins	BioCarta	*CASP6*	1,10,12,17
		CCNE1	1,9,12,17
		IGF1	1,2,7,17
		IL1a	2,7,8,15
		LTA	2,8,15,17
		C7	2,3,8,15
		IL6	2,3,7,15
		STAT4	3,8,10,17
	KEGG	*MOM6*	1,3,8,9,12,17
		RPA2	1,3,8,9,12,17
		POLA2	1,3,8,9,12
		PRIM2	1,3,8,9,12
		MOM2	1,3,8,9,12
		LTA	2,3,10,15,16
		SDHB	1,3,9,10,16
		POLE2	1,3,8,9,17
		COX7C	1,9,10,12,16
		COX8A	1,9,10,12,16
		NDUFB7	1,9,10,12,16
		NDUFA8	1,9,10,12,16
		NDUFA7	1,9,10,12,16
		SSBP1	1,8,9,12,17
	Reactome	*NUP93*	1,3,9,10,11,12,17
		POLE2	1,3,9,11,16,17
		POLR2L	1,3,9,11,12,16
		POLR2G	1,3,9,12,16,17
		PSMD14	1,3,9,11,12,17
		PSMC6	1,3,9,11,12,17
		RPA2	1,3,9,11,12,17
		NUP85	1.3.9.10.11.12
		NUP37	1.3.9.11.12.17
		NUP205	1.3.9.11.12.17
		CDC6	1.3.9.11.12.17
		CDC25A	1.3.9.11.12.17
		ORC4L	1.3.9.11.12.17
		KMCM6	1.3.9.11.12.17
		PRIM1	1.3.9,11,16,17
	Gene Ontology	n/a	n/a
Lower IQ Twins	BioCarta	n/a	n/a
	KEGG	*EP300*	1,3,6,12,13
		ACTN1	1,6,12,13,14
		PLOB2	1,3,8,13
		PIK30G	1,6,8,13
		CREBBP	1,3,12,13
		MAPK9	1,2,6,13
		LEF1	1,3,6,12
		PIK3R3	2,6,8,13
		AKR104	3,8,16,17
		PARD3	6,8,12,16
	Reactome	*MNAT1*	2,6,7,14
		RFC2	2,6,7,14
		PRIM1	2,6,7,14
		PRIM2	2,6,7,14
	Gene Ontology	*CHRNA2*	1,9,16,17
		KCNE2	1,9,16,17
		KCNQ3	1,9,16,17
		INHBA	1,9,14,17
		SLC34A3	1,5,16,17

n/a, no gene was shared by at least 4 twin pairs.
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demonstrated improved performance in various tests of learning and memory [39].

Identified by utilizing BioCarta pathway database, *IGF1* manifested up-regulation in higher IQ twins. The insulin-like growth factor (IGF) system is important in growth and development. While the exact mechanism remains unknown, the growth hormone (GH) and IGF-1 axis has been reported to play a role in the reduction of cognitive functions in aging population and patients with GH deficiency [20]. Methylation status of its promoter regions was studied, yet no difference was discerned between the twins. It is possible that *IGF1* is under some other epigenetic regulation, considering the actual mechanisms responsible for the cell type-specific expression patterns of this gene remain to be elucidated [40].

Since intelligence is a complex trait associated with many genes of small effect [41,42], it was not surprising that we failed to identify a single gene manifesting prevailing expression changes across all 17 twin pairs. We also noticed that by microarrays none of the candidate genes identified by methylation analyses was listed in the results of expression studies. A reason of the discrepancy of these two methods may be the lack of comprehensive accession to all known epigenetic regulations. DNA methylation at CpG sites across promoter regions has been deeply studied, while a number of other epigenetic regulatory mechanisms are also found to modulate gene expression [22]. Furthermore, ever since the genome-wide, single-base human DNA methylome mapping became possible, the correlation of methylation status of gene bodies and expression levels has been gaining attention [43]. It has been documented that DNA methylation of gene bodies is associated with gene activity. Therefore, it is possible that the intelligence-related expression profiles were subjected to this novel epigenetic regulation.

The present study has its conceptual and technical limitations. Conceptually, we hypothesized that changes in methylation status and expression levels could be captured in genomic DNA and total RNA extracted from whole blood and derivative lymphoblastic cell lines, respectively. Given the inability to probe DNA methylation status or gene expression in the human brain, except in postmortem studies, human blood is commonly used in transcriptional studies of various diseases including psychiatric disorders [44]. Although there is still no consensus regarding blood-based gene expression profiles as good surrogates for addressing neuroscientific research, the moderate correlation

between transcripts in whole blood and the central nervous system makes it an accessible alternative [45,46]. Several studies utilizing similar strategies to study twin pairs discordant for psychiatric disorders through comparisons of CpG islands methylation of peripheral blood cells or lymphoblast cell lines had detected a number of disease-associated epigenetic changes [47–49]. Moreover, it has been documented that the methylation changes of large-scale domains are linked to cell-specific differentiation [50]. Several functional gene sets we found (i.e., "cation channel activity", "cell-cell signaling", "extracellular region part", "G-protein coupled receptor protein signaling pathway", and "gated channel activity") were observed within neuronal highly methylated domains. The association between expression difference observed in lymphoblast cell lines and neuron-specific methylation patterns implies that these candidate gene sets are more likely to reflect the true differences in brains. Technical limitations of this study include low fold-change differences in expression levels manifested between co-twins. We did not carry out qRT-PCR for the genes identified by GSEA, considering that differences of less than 1.5-fold are thought to be beyond the limit of reproducibility [51].

Reverse causality should be considered in epigenetic studies, considering all known epigenetic marks are influenced by environmental exposures including diet, smoking, alcohol consumption, stress, or physical activities [52], It might be plausible that the changes we observed in this study resulted from the divergent lifestyle choices by subjects with different levels of intelligence, and not that these epigenetic changes caused the twins to differ.

Here, we presented the first study that used genome-wide epigenetic and transcriptomic profiling to identify epigenetic changes related to the discordance between MZ twins with normal-range intelligence. A list of new candidate genes possibly related to cognitive abilities was generated while further replications and functional analysis remain necessary.

Materials and Methods

Ethics statement

This study was conducted according to the principles expressed in the Declaration of Helsinki. Attendance was voluntary, and signed informed consent including information on genetic analyses was obtained from all participants. The Ethical Committees of Kobe University Graduate School of Medicine and Keio University Faculty of Letters approved study protocols.

Samples

A sub-sample of 326 twin pairs of twins from the Keio Twin Project were invited to Keio University, where the Kyodai Nx15-, one of the most often used group intelligence tests in Japan, was applied. The zygosity of participants was diagnosed by 15 polymorphic STR loci (AmpF, STR identifier kit, Applied Biosystems). Among the 240 pairs to have monozygosity, 34 MZ twin pairs who manifested differences in IQ score of more than 15 points between co-twins. One pair was excluded for a lower-than-normal IQ score (52 points). From the17 of the remaining 33 twin pairs, who agreed to participate in the study, peripheral blood was drawn and B-lymphoblastoid cell lines were established. For the treatment of 5-azadC (WAKO), a daily aliquot of 5 mM stock solution was added to flasks and thoroughly resuspended (final concentration of 1 mM). Cells were harvested after 3 days from the start of treatment.

Nucleic acids extraction

For human promoter microarrays and bisulfite genomic sequencing, genomic DNA was extracted from the blood via established methods. For gene expression microarrays and quantitative RT-PCR, total RNA was isolated using RNeasy Plus Mini kit (QIAGEN) from B-lymphoblastoid cell lines.

DNA methylation profiling

One microgram of genomic DNA was sonicated and subjected to methylated DNA enrichment using the MethylMiner methylated DNA enrichment kit (Invitrogen) as per the manufacturer's instructions. The methylated DNA fragments, amplified by the GenomePlex WGA reamplification kit 3 (SIGMA) and supplemented with dUTP, were further purified using the QIAquick PCR purification kit (QIAGEN).

According to Affymetrix's chromatin immunoprecipitation assay protocol, enriched methylated DNA was hybridized to GeneChip Human Promoter 1.0R arrays (Affymetrix), which comprised a coverage of over 25,500 human promoter regions.

AGCC (Affymetrix GeneChip Command Console)-format CEL files were first created, and then converted to GCOS (GeneChip Operating Software, Affymetrix)-format CEL files. For the pairwise analyses, paired CEL files were imported into MAT software to specify candidate regions (approximately 600 base pairs in length) with significantly different probe intensities between co-twins ($p<10^{-6}$).

To detect candidate loci across all 17 twin pairs, we utilized Partek Genomic Suite 6.5 software (Partek) to import the CEL files, and have the data converted to $\log_2$ values after normalized by the RMA (Robust Multichip Averaging) algorithm. After the signal from each probe for the higher-IQ sibling was subtracted from that of the lower-IQ co-twin across all probes, one-class t-test with statistical parameters set at $p<10^{-6}$ was carried out to detect significant regions.

Bisulfite sequencing

Genomic DNA was bisulfite-treated using the Methylcode bisulfite conversion kit (Invitrogen) as per the manufacturer's instructions. Amplification was performed with Takara LA Taq polymerase, with converted DNA-specific primers that were designed using MethPrimer. The amplicons were cloned into vectors using a TOPO TA cloning kit (Invitrogen). We performed direct sequencing of the plasmid DNA that was isolated using PI-200 auto-plasmid-isolator (KURABO) via the ABI 3730xl sequencing system (Applied Biosystems).

Quantitative RT-PCR

Two micrograms of the total RNA was subjected to reverse transcription using the SuperScript III first-strand synthesis system for RT-PCR (Invitrogen). Gene target amplifications, using Takara SYBR premix Ex Taq, were performed in triplicate in a matter of a serial 10 fold dilution. Housekeeping gene *GAPDH* served as the internal control gene. Mann-Whitney U test was performed to compare the relative expression levels between co-twins.

Gene expression profiling

We processed 300 nanograms of total RNA using the Ambion WT expression kit and the Affymetrix GeneChip WT terminal labeling kit according to the manufacturers' recommended methods. Hybridization and scanning of GeneChip Human Gene 1.0 ST arrays (Affymetrix), which comprised of more than 28,000 gene-level probe sets, were performed as per the manufacturer's

instructions. Partek Genomic Suite 6.5 software was used to import AGCC-format CEL files and normalize the data according to the RMA algorithm. ANOVA with an FDR-adjusted p set to 0.05 was used to determine those probe sets that were significantly different between the groups of twins with a higher IQ and their lower IQ co-twins. A one-class t-test analysis with multiple sample correction was conducted across all $\log_2$ ratios (higher-IQ twin/lower-IQ co-twin) for all 17 twin pairs. We also carried out pair-wise comparison for the expression array data and then included genes with a fold-change value more than 2. Genes replicated in the same tendency (up-regulated in the higher IQ twins, or up-regulated in the lower IQ twins) in most pairs were listed.

In another approach, the twins of each pair were categorized into higher and lower expression groups according to the expression level of every individual gene. A paired t-test was carried out to compare the mean IQ scores of the two groups. Corrected p of 10^{-6} was applied as the cutoff to define being positive.

GSEA was performed for functionally related genes across a spectrum of gene sets of C2 curated gene sets including BioCarta gene sets (217 gene sets), KEGG gene sets (186 gene sets), and Reactome gene sets (430 gene sets), and C5 GO gene sets (1,454 gene sets) separately. Pre-ranked gene lists, including lists with up-regulated/down-regulated genes in the group of twins with higher IQ scores sorted according to the p calculated by a between-group ANOVA test, and lists for each twin pair with genes sorted by between-sibling fold-change values, were constructed for the analyses. Gene sets with FDR q-value<0.25 after 1,000 permutation cycles were considered significantly enriched. Lists of leading edge subset genes, the cores of gene sets that account for the enrichment signal, were then generated. To create the list of enriched genes shared by plural twin pairs, the upper 100 enriched genes of each pair were included in the test.

Supporting Information

Dataset S1 Pair-wise comparison of expression array data. Genes with a fold-change value >2 were included. The positive value of fold-change designates up-regulation in the higher IQ twin, while the negative value designates the other way round. n/a indicates no gene matched the fold-change cutoff value.
(XLS)

Dataset S2 Pair-wise GSEA results using BioCarta database. Gene sets with a FDR q-value<0.25 were included. n/a indicates no gene set matched the FDR cutoff value.
(XLS)

Dataset S3 Pair-wise GSEA results using KEGG pathway database. Gene sets with a FDR q-value<0.25 were included. n/a indicates no gene set matched the FDR cutoff value.
(XLS)

Dataset S4 Pair-wise GSEA results using Reactome database. Gene sets with a FDR q-value<0.25 were included. n/a indicates no gene set matched the FDR cutoff value.
(XLS)

Dataset S5 Pair-wise GSEA results using GO database. Gene sets with a FDR q-value<0.25 were included. n/a indicates no gene set matched the FDR cutoff value.
(XLS)

Figure S1 Scatterplot of the 240 MZ twin pairs IQ scores. This diagram provides an overview of the IQ distribution for all 240 MZ twins from the Keio Twin Study. Each circle identifies one twin pair with its x-coordinate and y-coordinate representing, respectively, the IQ score of Twin A and Twin B. With the black line standing for regression, the correlation coefficient of 0.72 suggests the similarities between twins. Circles located outside of the space between two blue lines indicate twin pairs manifesting between-sibling IQ differences larger than 15 points and were considered to be recruited, while the red arrowhead points to one pair being excluded as a possible subject for a lower-than-normal IQ score.
(TIF)

Figure S2 Positive correlation between IQ scores differences and the number of loci different in methylation status. The positive correlation between the number of loci with significant differences in methylation patterns and the differences of IQ scores of the twin pairs was visualized. Twin pairs with larger differences in IQ scores tended to have more loci identified by screening for epigenetically regulated genes.
(TIF)

Figure S3 PCA results for the expression profiles of 17 twin pairs. Principle components ranked from the highest variance are named accordingly as PC 1st and PC 2nd. The PCA projection maps these two components data to 2 dimensions for visualization. In the scatter plots, each point represents a sample. The color of the symbol represents the relative IQ scores with red as higher and blue as lower. The number on each symbol indicates the twin pair ID. In contrast to the similarity between co-twins from each pair, no apparent gathering pattern could be recognized by the relative IQ scores.
(TIF)

Figure S4 Clustering analysis for the expression profiles of 17 twin pairs. Clustering analysis for the list of 644 genes manifesting more than a 1.1-fold change between the groups of twins with higher IQ scores and the groups of their co-twins under ANOVA analysis was performed. The number below each symbol indicates the twin pair ID. The color red and blue of the symbol indicate respectively the higher IQ and the lower IQ twin of each twin pair. The scale represents fold changes, according to the color map at the bottom of the figure. No apparent clustering could be recognized.
(TIF)

Table S1 Summary of candidate loci with methylation changes identified by promoter DNA methylation patterns and their bisulfite sequencing result.
(DOC)

Table S2 Up-regulated gene sets in the group of twins identified by GSEA (FDR q-value<0.25).
(DOC)

Table S3 Methylation profiling by bisulfite sequencing for IGF1 (Chr2:101335584□31398508) in Twin Pair ID 7.
(DOC)

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Author Contributions

Conceived and designed the experiments: CCY MF KK TT. Performed the experiments: CCY MF. Analyzed the data: CCY MF. Contributed reagents/materials/analysis tools: CS PCC JS HS KI TK JA. Wrote the paper: CCY MF KK TT.

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Large-scale replication and heterogeneity in Parkinson disease genetic loci

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Supplemental data at www.neurology.org

Supplemental Data

ABSTRACT

Objective: Eleven genetic loci have reached genome-wide significance in a recent meta-analysis of genome-wide association studies in Parkinson disease (PD) based on populations of Caucasian descent. The extent to which these genetic effects are consistent across different populations is unknown.

Methods: Investigators from the Genetic Epidemiology of Parkinson's Disease Consortium were invited to participate in the study. A total of 11 SNPs were genotyped in 8,750 cases and 8,955 controls. Fixed as well as random effects models were used to provide the summary risk estimates for these variants. We evaluated between-study heterogeneity and heterogeneity between populations of different ancestry.

Results: In the overall analysis, single nucleotide polymorphisms (SNPs) in 9 loci showed significant associations with protective per-allele odds ratios of 0.78–0.87 (*LAMP3*, *BST1*, and *MAPT*) and susceptibility per-allele odds ratios of 1.14–1.43 (*STK39*, *GAK*, *SNCA*, *LRRK2*, *SYT11*, and *HIP1R*). For 5 of the 9 replicated SNPs there was nominally significant between-site heterogeneity in the effect sizes (I^2 estimates ranged from 39% to 48%). Subgroup analysis by ethnicity showed significantly stronger effects for the *BST1* (rs11724635) in Asian vs Caucasian populations and similar effects for *SNCA*, *LRRK2*, *LAMP3*, *HIP1R*, and *STK39* in Asian and Caucasian populations, while *MAPT* rs2942168 and SYT11 rs34372695 were monomorphic in the Asian population, highlighting the role of population-specific heterogeneity in PD.

Conclusion: Our study allows insight to understand the distribution of newly identified genetic factors contributing to PD and shows that large-scale evaluation in diverse populations is important to understand the role of population-specific heterogeneity. ***Neurology*® 2012;79:659–667**

GLOSSARY

CI = confidence interval; **GEO-PD** = Genetic Epidemiology of Parkinson's Disease; **GWAS** = genome-wide association studies; **HWE** = Hardy-Weinberg equilibrium; **MALDI-TOF** = matrix-assisted laser desorption/ionization time-of-flight; **MSA** = multiple system atrophy; **OR** = odds ratio; **PD** = Parkinson disease; **SNP** = single nucleotide polymorphism.

Genome-wide association studies (GWAS) have provided tangible gains in understanding the genetic architecture of complex diseases,[1,2] including Parkinson disease (PD).[3] Several GWAS have been conducted in PD in Caucasian populations and only 1 in the Asian population.[3–11] Consistent and reproducible association signals were confirmed in α-synuclein (*SNCA*), leucine-rich repeat kinase 2 (*LRRK2*), and microtubule-associated protein tau (*MAPT*), thus underscoring the importance of these 3 genes in the pathophysiology of the common sporadic forms of PD.[3–10,12] In addition to that, different studies have provided some evidence for an association for *BST1*, *GAK*, and *HLA-DRB5* with PD.[6–9,13]

A recently published GWAS meta-analysis in PD increased the number of identified PD genetic loci to 11.[14] This study reported significant between-study heterogeneity for some of the 11 genetic loci[14] even though data were restricted to Caucasian descent populations.

It is important to establish whether the 11 genetic loci that have been postulated to be associated with PD are replicated when tested with direct genotyping in a larger spectrum of diverse populations. The consistency or lack thereof of the genetic effects of these genetic

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variants across different populations may help to determine whether they represent genuine loci for PD susceptibility and whether they can be used for risk prediction across these diverse populations.[15] To gain further insight into genetic factors contributing to PD across different populations and define the implications of between-population heterogeneity, we performed a large-scale replication study within the GEO-PD consortium.

METHODS

Consortium. Investigators from the Genetic Epidemiology of PD (GEO-PD) Consortium were invited to participate in this study. A total of 21 sites representing 19 countries from 4 continents agreed to contribute DNA samples and clinical data for a total of 17,705 individuals (8,750 cases and 8,955 controls). Healthy individuals matched for age and gender served as controls. They underwent neurologic examination and were excluded from the study whenever there was clinical evidence for any extrapyramidal disorder.

Genotyping. We selected 1 SNP per each gene locus, exactly as they were proposed by the recently published GWAS meta-analysis.[14] Genotyping was performed by a central genotyping core (Department of Human Genetics, Helmholtz Zentrum, Munich). Each site provided 100–200 ng of DNA to the laboratory core. In total 11 SNPs located in and around the genes encoding *SYT11*, *ACMSD*, *STK39*, *LAMP3*, *GAK*, *BST1*, *SNCA*, *HLA-DRB5*, *LRRK2*, *HIP1R*, and *MAPT* were genotyped. The genotyping core was blinded to case-control status of each site. Genotyping was performed using a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry on a MassArray system (Sequenom, San Diego, CA). Cleaned extension products were analyzed by a mass spectrometer (Bruker Daltronik, USA) and peaks were identified using the MassArray Typer 4.0.2.5 software (Sequenom). Assays were designed by the AssayDesigner software 4.0 (Sequenom) with the default parameters for the iPLEX Gold chemistry and the Human GenoTyping Tools ProxSNP and PreXTEND (Sequenom). All variants were genotyped in 1 multiplex assay. An experienced investigator blinded to case or control status of the samples visually checked genotype clustering. The average call rate of the variants was >97%.

In order to further enrich the samples of Asian ancestry populations, we also included GWAS data from a Japanese population (988 cases, 2,521 controls).[6] We used r^2 threshold of 0.8–1.0 to select proxy SNPs from the Japanese GWAS. Using this threshold, we were able to capture only 3 SNPs from *BST1*, *SNCA*, and *LRRK2* genes.

Standard protocol approvals, registrations, and patient consents. The local Ethics Committee approved the study. All participants signed an informed consent.

Analysis. An exact test was used to assess whether the genotype distributions for each SNP deviated from Hardy-Weinberg equilibrium (HWE) among controls; each site was tested separately and deviation from HWE was considered significant at <0.01. We excluded data from sites where the missing rate was >5%. For our analysis, we adhered to the same allele coding as in the previous GWAS meta-analysis.[14]

For consistency effect estimates based on minor vs major allele contrast were computed. We used an additive model adjusted for age and gender to obtain effect estimates. Results were then synthesized using fixed and random effects models. Fixed effect models assume that the genetic effect is the same in populations from different sites and that observed differences are due to chance alone. For associations showing between-study heterogeneity, fixed effect estimates yield narrower confidence intervals (CIs) and smaller p values as compared to random effects models, which incorporate between-study heterogeneity.[16–18] Fixed effects analysis tests the null hypothesis of no association in all studied populations that are analyzed. Routinely, this assumption is used in GWAS settings to increase the power of meta-analysis to detect associations that may exist in some (at least 1) population. However, in presence of heterogeneity the effects may differ substantially in different populations and not all populations may show a genetic effect for the variant of interest. Random effects models allow the genetic effects might be different due to genuine heterogeneity that may exist across different sites. Random effects calculations take into account the estimated between-study heterogeneity. We used the inverse variance method for fixed effects models. Cochran Q test of homogeneity and the I^2 metric were used to evaluate the between-site heterogeneity. The Q statistics follows χ^2-based distribution with k − 1 degrees of freedom (k = number of studies). I^2 is estimated by the ratio (Q-df)/Q, where *df* is degrees of freedom. The I^2 metric ranges from 0% to 100% and measures the proportion of variability that is beyond chance. Typically estimates of I^2 <25% are considered to reflect little or no heterogeneity, 25%–50% moderate heterogeneity, 50%–75% large heterogeneity, and >75% very large heterogeneity. It should be acknowledged that I^2 can have large uncertainty in its estimation especially for variants with low minor allele frequency. Therefore, we also estimated the 95% CI of I^2.[17]

The overall main analysis considered all sites and populations irrespective of ancestry. Then, we separately analyzed Caucasian and Asian sites and we compared the genetic effects in these 2 major ancestry groups.

The SNPs evaluated in the recently published GWAS meta-analysis are common with minor allele frequencies varying from 13% to 46%,[14] except for SNP, rs34372695 (SYT11) where the minor allele frequency is 2%. Therefore, based on minor allele frequency and effect estimates obtained in the GWAS meta-analysis,[14] power calculations showed that our study would have at least 99% power to detect an allele-based odds ratio (OR) of 1.2 for minor allele frequencies of 10% or higher for $\alpha = 0.05$. Based on genome-wide significance level ($\alpha = 5 \times 10^{-8}$), our study would have 43% power to detect an allele-based OR of 1.2 for minor allele frequency of 10%, but it would be 99% for same minor allele frequency and an OR of 1.4. Power would be only 69% for a minor allele frequency of 2% and OR of 1.2, but it would be 99% for the same minor allele frequency of 2% and an OR of 1.5.

Meta-analyses were performed using STATA 9.0 (Stata Corp., College Station, TX) and Review Manager 4.2.7. p Values are 2-tailed.

RESULTS

Characteristics of sites and overall database. Twenty-one sites contributed a total of 8,750 cases and 8,955 controls. Characteristics of all participating sites are shown in table 1. Most sites contributed participants of Caucasian ancestry (n = 16); 5 sites (counting also the GWAS performed in the Jap-

Table 1 Description of datasets contributed by each study site

Site	Country	No.	Case	Control	Male (%)	Female (%)	Mean AAO	Mean age at study	Diagnostic criteria
Annesi	Italy	394	197	197	204 (51.7)	190 (48.2)	61.5	63.7	UKPDBB
Brice[a]	France	505	272	233	302 (59.8)	203 (40.1)	47.6	57.8	UKPDBB
Bozi	Greece	222	114	108	107 (48.1)	115 (51.8)	69.9	74.5	UKPDBB
Wszolek	US	1,518	692	826	794 (52.3)	724 (47.6)	64.4	71.7	UKPDBB
Garraux	Belgium	82	68	14	45 (54.8)	37 (45.1)	62.1	69.6	UKPDBB
Hadjigeorgiou	Greece	714	357	357	379 (53.0)	335 (46.9)	63.4	63.7	UKPDBB
Jeon	Korea	749	408	341	314 (41.9)	435 (58.0)	57.6		UKPDBB
Opala	Poland	629	352	277	340 (54.0)	288 (45.7)	50.2	68.1	UKPDBB
Lynch	Ireland	740	368	372	340 (45.9)	400 (54.0)	50.5	70.7	UKPDBB
Lin	Taiwan	320	160	160	160 (50)	160 (50)	62.0	70.8	UKPDBB
Facheris	Italy	181	114	67	86 (47.5)	95 (52.4)	63.0		UKPDBB
Maraganore	US	1,024	801	223	600 (58.5)	361 (35.3)	59	74.7	Bower
Mellick	Australia	2,024	1,012	1,012	1,042 (51.4)	981 (48.4)	59	72.2	Bower
Morrison[a]	England	1,120	766	354	606 (54.1)	514 (45.8)	66.1		UKPDBB
Mok	China	436	260	176	264 (60.5)	170 (38.9)		63.5	UKPDBB
Aasly	Norway	1,278	656	622	721 (56.4)	557 (43.5)	58.8	72.9	UKPDBB
Wirdefeldt	Sweden	299	83	216	147 (49.1)	152 (50.8)	65.8	71.4	Gelb
Van Broeckhoven	Belgium	1,010	501	509	500 (49.5)	509 (50.3)	60.5	66.3	Pals/Gelb
Rogaeva	Canada	560	387	173	303 (54.1)	257 (45.8)	49.7	64.2	UKPDBB
Tan	Singapore	391	194	197	244 (62.4)	147 (37.5)	59.7	54.0	UKPDBB
Toda	Japan	3,509	988	2,521	1,844 (52.6)	1,665 (47.4)	58.7	66.0	UKPDBB
Total		17,705	8,750	8,955			59.5	67.6	

Abbreviations: AAO = age at onset; GWAS = genome-wide association studies; UKPDBB = UK Parkinson's Disease Brain Bank.
[a] Also included in the previously published GWAS.[7,8]

anese population[9]) included participants of Asian ancestry. We excluded 1 site with 114 cases and 67 controls from the analysis due to a plate layout error. The median age at onset was 59 years and median age at examination was 67 years.

We observed that for one site, effect estimates for all SNPs were "inverse" as compared to other Caucasian sites. Allele flipping for one particular site in the same Caucasian descent might reflect error in sampling ascertainment and is unlikely to reflect genuine effects.[19] This site (n = 181) was therefore excluded from further analyses. Overall, genotype call rates were >97%. The genotype distribution for each SNP in the controls of each site showed no departure from HWE, except for rs6599388 (*GAK*) in samples from 4 Asian sites. We therefore excluded this SNP (rs6599388) from analyses in the Asian population.

Overall data synthesis. We observed consistent and reproducible associations for *SNCA*, *LRRK2*, *MAPT*, *BST1*, *GAK*, *STK39*, *SYT11*, *LAMP3*, and *HIP1R* loci but not for *ACMSD* (rs10928513) or *HLA-DRB5* (rs3129882) where the per-allele OR was very close to the null (1.02 and 0.95, respectively) and statistically nonsignificant (table 2). Thus we provide unequivocal support for the involvement of these newly identified genetic loci in the pathogenesis of PD.

Summary effect estimates were generally comparable with the previous GWAS meta-analysis results (table 2), although effect estimates in this study were stronger for STK39 and somewhat weaker for *LRRK2* compared to the previous GWAS meta-analysis.[14] Exclusion of 1,625 samples that overlap with the previously published GWAS did not change any of the estimates (table e-1 on the *Neurology*® Web site at www.neurology.org). The protective per-allele OR ranged from 0.78 to 0.87 (*LAMP3*, *BST1*, and *MAPT*) and the susceptibility per-allele OR ranged from 1.14 to 1.43 (*STK39*, *GAK*, *SNCA*, *LRRK2*, *SYT11*, and *HIP1R*). Cochran Q statistics were nominally significant for *STK39*, *LAMP3*, *BST1*, and *SNCA* with I^2 estimates ranging from 39% to 48%. The heterogeneity reflected primarily differences in the magnitude of the effect sizes across different sizes, while the direction of the effect was consistent in all sites, with rare exceptions.

Table 2 Overall analysis irrespective of ethnicity and influence of between-study heterogeneity

Gene	SNP	Q test p value	I^2 (95% CI)	Odds ratio (95% CI) by random effects	Fixed effects p value	Random effects p value	IPDGC study odds ratio (fixed effects p values)
ACMSD	rs10928513	0.71	0 (0–54)	1.02 (0.96–1.08)	0.479	0.479	1.07 (0.003)
STK39	rs2102808	0.02	46 (0–68)	1.21 (1.08–1.35)	0.0001	0.001	1.12 (0.0016)
LAMP3	rs11711441	0.04	39 (0–64)	0.85 (0.77–0.94)	3.01×10^{-5}	0.002	0.87 (6.92×10^{-5})
GAK	rs6599388	0.06	39 (0–64)	1.19 (1.10–1.28)	3×10^{-6}	0.001	1.14 (7.46×10^{-8})
HLA-DRB5	rs3129882	0.23	18 (0–53)	0.95 (0.90–1.01)	0.12	0.15	0.80 (9.3×10^{-8})
BST1	rs11724635	0.02	43 (0–65)	0.87 (0.83–0.91)	2.01×10^{-6}	0.00001	0.87 (2.43×10^{-9})
SNCA	rs356219	0.02	48 (0–69)	1.30 (1.21–1.40)	4.23×10^{-23}	4.23×10^{-23}	1.27 (4.23×10^{-23})
SYT11	rs34372695	0.38	6 (0–50)	1.43 (1.15–1.78)	0.001	0.001	1.44 (1.18×10^{-6})
LRRK2	rs1491942	0.75	0 (0–50)	1.14 (1.07–1.21)	1.06×10^{-8}	1.06×10^{-8}	1.30 (1.06×10^{-8})
HIP1R	rs10847864	0.90	0 (0–44)	1.15 (1.09–1.21)	9.06×10^{-7}	9.06×10^{-7}	1.13 (9.06×10^{-7})
MAPT	rs2942168	0.14	29 (0–62)	0.78 (0.71–0.85)	1.37×10^{-13}	1.37×10^{-13}	0.80 (1.37×10^{-13})

Abbreviations: CI = confidence interval; IPDGC = International Parkinson disease Genomics Consortium; SNP = single nucleotide polymorphism.

Analysis including only Caucasian sites. Restricting the analysis to Caucasian sites only resulted in per-allele ORs that ranged from 0.78 to 0.90 for the 3 replicated protective loci (*BST1*, *LAMP3*, and *MAPT*) and from 1.14 to 1.43 for the 6 replicated susceptibility loci (*STK39*, *GAK*, *SNCA*, *LRRK2*, *SYT11*, and *HIP1R*), while *ACMSD* and *HLA-DRB5* still had no significant effect (table 3 and figure e-1).

Summary effect estimates were generally comparable to those of the previous GWAS meta-analysis,[14] except for modest differences in STK39 and LRRK2 effect sizes, as noted above also for the overall analysis. There was nominally significant heterogeneity only for SNCA and LAMP3 (I^2 estimates 51% and 46%, respectively), but this reflected primarily differences in the magnitude of the effect size estimates rather than direction of effects across sites (figure e-1).

Analysis including only Asian sites. In the Asian series, not only the *SYT11* SNP, but also the *ACMSD* and *MAPT* SNPs were monomorphic (table e-2). Summary effect estimates for the remaining SNPs are shown in table 4. We again observed consistent nominally significant evidence of association for all loci except for *STK39* (which still had an effect size estimate consistent with what was seen in the overall analysis) and *HLA-DRB5* (which had a point estimate very close to the null), which still had an effect size estimate consistent with what was seen in the overall analysis. Results were generally consistent across sites, with the exception of *STK39* that showed very large heterogeneity ($I^2 = 73\%$) (figure e-2).

Comparison of effect size estimates. Five gene loci (*HIP1R*, *LAMP3*, *LRRK2*, *SNCA*, and *STK39*)

Table 3 Caucasian specific effect estimates and influence of between-study heterogeneity

Gene	SNP	Q test p value	I^2 (95% CI)	Odds ratio (95% CI) by random effects	Fixed effects p value	Random effects p value
ACMSD	rs10928513	0.71	0 (0–54)	1.02 (0.96–1.08)	0.479	0.479
STK39	rs2102808	0.10	37 (0–65)	1.21 (1.08–1.35)	0.000	0.001
LAMP3	rs11711441	0.02	46 (0–70)	0.86 (0.76–0.97)	0.001	0.025
GAK	rs6599388	0.27	17 (0–57)	1.14 (1.06–1.23)	1.01×10^{-4}	0.001
HLA-DRB5	rs3129882	0.10	32 (0–63)	0.95 (0.88–1.02)	0.11	0.16
BST1	rs11724635	0.14	29 (0–62)	0.90 (0.85–0.95)	0.001	0.003
SNCA	rs356219	0.01	51 (0–72)	1.30 (1.19–1.42)	4.23×10^{-23}	4.23×10^{-23}
SYT11	rs34372695	0.38	6 (0–50)	1.43 (1.15–1.78)	0.001	0.001
LRRK2	rs1491942	0.80	0 (0–47)	1.15 (1.07–1.23)	1.06×10^{-8}	1.06×10^{-8}
HIP1R	rs10847864	0.90	0 (0–47)	1.15 (1.08–1.22)	9.06×10^{-7}	9.06×10^{-7}
MAPT	rs2942168	0.14	29 (0–62)	0.78 (0.71–0.85)	1.37×10^{-13}	1.37×10^{-13}

Abbreviations: CI = confidence interval; SNP = single nucleotide polymorphism.

Table 4 Asian specific effects and influence of between-study heterogeneity

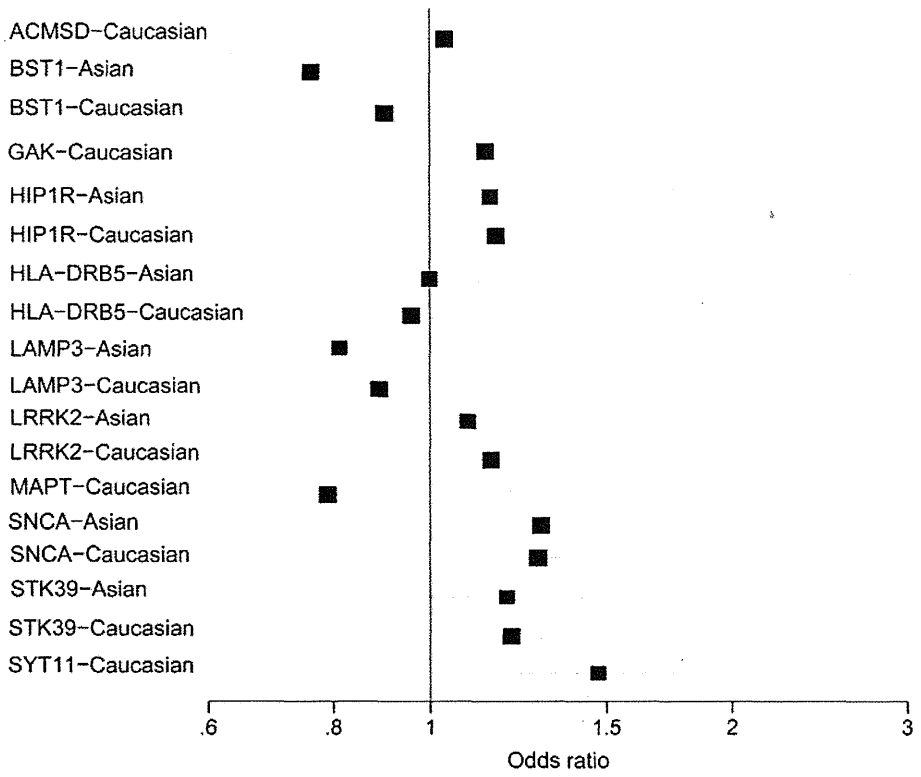
Gene[a]	SNP	Q test p value	I^2 (95% CI)	Odds ratio (95% CI) by random effects	Fixed effects p value	Random effects p value
STK39	rs2102808	0.01	73 (0–88)	1.14 (0.85–1.52)	0.28	0.37
LAMP3	rs11711441	0.33	12 (0–72)	0.81 (0.67–0.97)	0.01	0.03
BST1	rs11724635	0.07	53 (0–81)	0.74 (0.68–0.81)	1.2×10^{-6}	0.001
HLA-DRB5	rs3129882	0.81	0 (0–68)	0.98 (0.85–1.13)	0.85	0.85
SNCA	rs356219	0.49	0 (0–68)	1.24 (1.08–1.43)	0.002	0.002
LRRK2	rs1491942	0.31	16 (0–73)	1.13 (1.02–1.24)	0.005	0.01
HIP1R	rs10847864	0.36	6 (0–70)	1.14 (0.99–1.32)	0.05	0.06

Abbreviations: CI = confidence interval; HWE = Hardy-Weinberg equilibrium; SNP = single nucleotide polymorphism.
[a] ACMSD, SYT11, MAPT SNPs were monomorphic; GAK SNP showed departure from HWE and thus is not included in the table.

where both Caucasians and Asian populations were represented showed no difference in effect size estimates that were different beyond chance (figure 1). Conversely for *BST1*, the effects were different beyond chance for Asian and Caucasian populations with stronger genetic effects in the former.

DISCUSSION We performed a large-scale evaluation to assess the role of recently discovered genetic risk variants in the pathogenesis of PD in different populations. Our study confirms 9 of the 11 postulated susceptibility SNPs for PD. The confirmed SNPs include the previously well documented *LRRK2*, *SNCA*, *BST1*, *GAK*, and *MAPT* associations,[3,6–8,10,20] and 4 of the 5 more recently proposed associations in *STK39*, *SYT11*, *LAMP3*, and *HIP1R* SNPs that might act as risk factors for PD. Conversely, we were unable to confirm the association between PD risk and SNPs rs10928513, rs3129882 in *ACMSD* and *HLA-DRB5*, respec-

Figure 1 Forest plot showing the comparison of effect of each single nucleotide polymorphism (SNP) in Caucasian and Asian population



Boxes indicate the summary effect estimate. SNPs in 3 loci (MAPT, SYT11, and ACMSD) were monomorphic in the Asian population and thus are not included in the graph. GAK SNP showed deviation from Hardy-Weinberg equilibrium in Asian series and thus excluded from the graph.

tively. Three SNPs were monomorphic in the Asian datasets.

The recently proposed *ACMSD* locus is likely to have represented a spurious association. The OR is very close to the null and 95% CI also excludes an OR larger than 1.08. Of note, even in GWAS meta-analysis, the OR estimate was only 1.07 in the replication phase, as compared with 1.38 in the discovery phase. Our results are in agreement with a recently published study.[21] This finding suggests that large-scale replication with direct genotyping is useful even for SNPs that pass conventional genome-wide significance thresholds. It is possible that with the use of the more extended imputation platforms using the 1,000 Genomes Project, the number of comparisons made is larger than what was done in the past with more limited imputation platforms and this may thus require more stringent levels of significance to claim genome-wide significance.

As observed in a recently published study,[9] the frequency of the *HLA-DRB5* SNP, rs3129882, varies considerably even within seemingly homogenous Caucasian populations; the frequency of risk allele is low in subjects of Northern European descent as compared to subjects from Southern European descent. Therefore, the observed lack of association for the *HLA-DRB5*-specific SNP rs3129882 should be interpreted with caution. Moreover, directionality as well as the magnitude of effect estimates obtained in our study for the *HLA-DRB5* locus specific SNP are comparable with previously published studies.[9,22]

Our study shed light on the role of heterogeneity in PD genetics. Detection of heterogeneity could provide new insight to understand the genetic architecture of the disease.[17,23,24] A number of factors can be attributed to the observed heterogeneity. First, clinically overlapping pathologies may lead to heterogeneity. Indeed the presence of distinct subgroups of patients during early clinical stages of PD could contribute to clinical heterogeneity.[25] Therefore, it is worth it to consider that genetic variants may exert different pathologic processes that eventually lead to complex clinical phenotypes.[26] For example, it has been shown that multiplications of *SNCA* gene lead to clinical phenotype, which clinically overlap with multiple system atrophy (MSA).[27] Moreover, SNPs in the 3′UTR of *SNCA* were shown to be associated with PD as well as MSA.[3,6–8,28] The most significant SNPs in both diseases clustered around 3≪UTR of *SNCA*; SNP rs11931074, that was significantly associated with MSA in contrast to rs356219 in the PD meta-analysis.[14,28] The r^2 between these 2 markers was only 0.16 in the Caucasian population. These 2 distinct signals in different yet overlapping pathologies therefore might reflect one cause of genetic heterogeneity. Second, heterogeneity might reflect that different tagging polymorphisms were used in previously published GWAS. Of note, a recently published GWAS from United Kingdom, France, and Netherlands provided weak (as they did not surpass genome-wide significant threshold) yet consistent association signals for the *BST1* and *GAK* gene.[7,8,13] This probably reflects that the investigated markers were not causal variants but in linkage disequilibrium with a potential causal variant across different studies.

Our study helps to understand the role of population-specific heterogeneity in PD risk loci. Some risk variants exist only in populations of specific ancestry. For example, it is known that an ancient inversion (~900 kb) in the *MAPT* region occurred, which led to the formation of 2 nonrecombining haplotypes, H1 and H2.[29] The H2 haplotype is absent in East Asian populations as has been shown also by a recently published Asian GWAS that revealed no association for the *MAPT* locus.[6] This is in contrast to previously published candidate gene studies and GWAS in Caucasian populations that have shown consistent association with the *MAPT* locus.[3,7,8,13]

Some other variants may have a different magnitude of effect in populations of different ancestry. In our data, this is well exemplified by *BST1*, where the OR was significantly larger in populations of Asian than those of Caucasian ancestry. Conversely, we found that associations in 5 loci (*SNCA*, *LRRK2*, *LAMP3*, *HIP1R*, and *STK39*) had similar effects in these 2 ancestries.

The consistency or diversity of effect sizes in identified associations may reflect different patterns of linkage disequilibrium in these loci in diverse populations. It may also reflect differences in the susceptibility to develop PD and perhaps also differences in age at onset in different ancestry groups. The diversity in the magnitude of effects is important to take into account when considering the use of such information for personalized risk modeling.[30] Therefore caution should be used in extrapolating risks across different populations.

Our study provides strong and independent support for the role of 9 loci in the pathogenesis of PD in different populations. The detection and documentation of heterogeneity across different populations is useful in understanding the genetic architecture of this complex disease and in properly framing our ability to use this information in different clinical populations.

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SHORT REPORT

A multi-centre clinico-genetic analysis of the VPS35 gene in Parkinson disease indicates reduced penetrance for disease-associated variants

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ABSTRACT

Background Two recent studies identified a mutation (p.Asp620Asn) in the vacuolar protein sorting 35 gene as a cause for an autosomal dominant form of Parkinson disease . Although additional missense variants were described, their pathogenic role yet remains inconclusive.

Methods and results We performed the largest multi-center study to ascertain the frequency and pathogenicity of the reported vacuolar protein sorting 35 gene variants in more than 15,000 individuals worldwide. p.Asp620Asn was detected in 5 familial and 2 sporadic PD cases and not in healthy controls, p.Leu774Met in 6 cases and 1 control, p.Gly51Ser in 3 cases and 2 controls. Overall analyses did not reveal any significant increased risk for p.Leu774Met and p.Gly51Ser in our cohort.

Conclusions Our study apart from identifying the p. Asp620Asn variant in familial cases also identified it in idiopathic Parkinson disease cases, and thus provides genetic evidence for a role of p.Asp620Asn in Parkinson disease in different populations worldwide.

INTRODUCTION

There is increasing interest to try to identify uncommon and rare genetic variants that increase the risk of common diseases and that are difficult to identify using traditional genome-wide association studies (GWAS) approaches.[1] Rare variants which are not mapped by GWAS can be identified by using next generation sequencing, that is, exome sequencing in large families with multiple affected individuals.[2] Exome sequencing is now routinely used to identify rare mutations in familial forms of disease in diverse phenotypes.[2]

Two recent studies independently performed exome sequencing in large families of Caucasian descent, and identified a mutation in the vacuolar protein sorting 35 (VPS35) gene as a possible cause for an autosomal dominant form of Parkinson disease (PD).[3 4] In addition, several non-synonymous base exchanges were identified, but their involvement in disease pathogenesis remains inconclusive. Furthermore, recently published studies provided conflicting results regarding the role of VPS35 in PD.[5–8] Here, we performed a large multi-centre study to determine the frequency and pathogenicity of VPS35 variants in PD in diverse populations worldwide.

METHODS

Consortium

Investigators from the Genetic Epidemiology of Parkinson disease Consortium were invited to participate in this study. A total of 23 sites representing 19 countries from four continents agreed to contribute DNA samples and clinical data for a total of 15 383 individuals (8870 cases and 6513 controls). Control individuals underwent neurological examination and were excluded from the study whenever there was clinical evidence for any extrapyramidal disorder.

Genotyping

We selected seven non-synonymous variants exactly as they were proposed.[3] In addition, we selected tag single nucleotide polymorphisms

(SNPs) (HapMap Rel 28 phase II+III, Aug10, National Centre for Biotechnology Information. B36 dbSNP b126; http://www.hapmap.org) that cover the common genetic variants in the VPS35 gene using an r^2 threshold of 0.8–1.0 to select tag SNPs for VPS35 gene. Using this strategy, we were able to capture 23 SNPs in a 40 kb region, including VPS35 ('chr16:46 693 589–46 723 144 based on hg 19'). Therefore, in total, 10 SNPs located in the VPS35 were genotyped (including seven rare non-synonymous and three common variants). Genotyping was performed by a central genotyping core. Genotyping was performed using a matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry on a MassArray system (Sequenom, San Diego, California, USA). Cleaned extension products were analysed by a mass spectrometer (Bruker Daltronic, Billerica, MA, USA) and peaks were identified using the MassArray Typer 4.0.2.5 software (Sequenom). Assays were designed by the AssayDesigner software 4.0 (Sequenom) with the default parameters for the iPLEX Gold chemistry and the Human GenoTyping Tools ProxSNP and PreXTEND (Sequenom). All variants were genotyped in one multiplex assay. The average call rate of the variants was >97%. The local Ethics Committee approved the study. All participants gave signed informed consent.

Statistical analysis

Logistic regression was used to test the association between VPS35 and PD in our overall cohort. For common variants (minor allele frequency >5%), we synthesised the effect estimates using fixed and random effects models. Fixed effect models assume that the genetic effect is the same in populations from different sites and that observed differences are due to chance alone. For associations showing between-study heterogeneity, fixed effect estimates yield narrower CIs and smaller p values as compared with random effects models, which incorporate between-study heterogeneity.[9 10] Random effects models allow the genetic effects might be different due to genuine heterogeneity that may exist across different sites. Random effects calculations take into account the estimated between-study heterogeneity. Cochran's Q test of homogeneity and the I^2 metric were used to evaluate the between-site heterogeneity. The I^2 metric ranges from 0% to 100% and measures the proportion of variability that is beyond chance. Typically, estimates of I^2<25% are considered to reflect little or no heterogeneity, 25%–50% moderate heterogeneity, 50%–75% large heterogeneity and >75% very large heterogeneity. The overall main analysis considered all sites and populations irrespective of ancestry. For variants with minor allele frequency <1%, an exact test was used to compare the frequency differences between cases and controls combining data across all 21 sites.

RESULTS

Characteristics of sites and overall database

Overall, 23 sites contributed a total of 8870 cases and 6513 controls. Characteristics of all participating sites are shown in table 1. Most sites contributed participants of Caucasian ancestry (N=19); four sites included participants of Asian ancestry. The proportion of men and women ranged from 42% to 58% across different participating sites (table 1). The median age at onset of PD in our studied population was 61 years.

Rare variants

Overall, we observed p.Asp620Asn in seven cases, p.Leu774Met in six cases and one control, p.Gly51Ser in three cases and two controls. Details per site are shown in table 2. The controls subjects carrying p.Leu774Met (P-13) and p.Gly51Ser (P-2 and P-16) at the time of study sampling were 81, 84 and 76 years, respectively. In Caucasian populations, the number of carriers in cases and controls for the three variants were 5 versus 0 (pAsp620Asn), 4 versus 1 (pLeu774Met) and 3 versus 1 (p.Gly51Ser), respectively. In Asian descent populations, the respective numbers were 2 versus 0 (p.Asp620Asn), 2 versus 0 (p.Leu774Met) and 0 versus 1 (p.Gly51Ser). Most interestingly, two out of seven patients carrying the p.Asp620Asn variant presented without any family history for PD. This represents the first evidence for reduced penetrance of the respective variant initially attributed to autosomal dominant familial PD. We did not observe any carriers for one variant (p.Arg524Trp) in our cohort. Two non-synonymous variants (p.Met57Ile, p.Thr82Arg) failed genotyping. By collapsing the rare variants across different sites, we did not observe statistically significant increased risk for p.Leu774Met and p.Gly51Ser in our cohort (see online supplementary table S1).

Overall data synthesis for common variants

Out of three tag SNPs, one SNP (rs3218745) failed genotyping. We did not observe significant association for any of common variants with PD either with either fixed effect or random effect models (see online supplementary table S2). The OR ranged from 0.96 to 0.99 and tight 95% CIs excluded modest association effects. We observed no substantial heterogeneity for the two genotyped SNPs, and also the Q test was non-statistically significant for common SNPs. Moreover, examining the Caucasian or Asian populations separately did not change our results (data not shown).

Clinical features

All PD patients who carried potential pathogenic variants (p.Asp620Asn, p.Gly51Ser, p.Leu774Met) were clinically diagnosed with PD (Online supplementary clinical analysis data). A few of these (0.2%) affected individuals also have a positive family history. Affected individuals exhibited classical symptoms of PD (resting tremor, bradykinesia, rigidity) (table 2). The clinical diagnosis of PD was made by movement disorder specialists who used UK brain bank criteria for PD. Non-motor symptoms were present in the majority of PD patients carrying a pathogenic variant (table 2). Interestingly, hallucinations and dementia were also observed in one asymptomatic carrier suggesting clinical heterogeneity associated with VPS35. The identified healthy carriers have not shown any sign of PD as yet (table 2).

DISCUSSION

We performed the first multi-centre study to define the role of the VPS35 gene (PARK17) in PD by assessing the frequency of the reported non-synonymous variants in familial and sporadic PD patients from different populations worldwide. Among 15 383 subjects genotyped, we found a pathogenic relevance for p.Asp620Asn in different populations. Most interestingly, out of seven subjects who carry p.Asp620Asn, two have a negative family history. Therefore, our results provide additional evidence that VPS35 is a rare cause of familial as well as the common sporadic form of PD. In total, about 0.4% of PD cases in diverse population were due to disease-associated variant in the VPS35 gene. Our lack of supporting the role of common variants of the VPS35 gene in PD is consistent with recently published GWAS and also meta- analyses of GWAS of PD, as none of these highlighted the role of common variability in VPS35 gene as a risk factor for PD.[11–15] The p.Asp620Asn

Table 1 Description of datasets contributed by each study site

Site	Country	N	Case	Control	Male (%)	Female (%)	Mean AAO	Mean Age at study	Diagnostic criteria
Annesi	Italy	394	197	197	204 (51.7%)	190 (48.2%)	61.5	63.7	UKPDBB
Brice	France	505	272	233	302 (59.8%)	203 (40.1%)	47.6	57.8	UKPDBB
Bozi	Greece	222	114	108	107 (48.1%)	115 (51.8%)	69.9	74.5	UKPDBB
Wszolek	USA	1518	692	826	794 (52.3%)	724 (47.6%)	64.4	71.7	UKPDBB
Garraux	Belgium	82	68	14	45 (54.8)	37 (45.1%)	62.1	69.6	UKPDBB
Hadjigeorgiou	Greece	714	357	357	379 (53.0%)	335 (46.9%)	63.4	63.7	UKPDBB
Jeon	Korea	749	408	341	314 (41.9%)	435 (58.0%)	57.6	NA	UKPDBB
Opala	Poland	629	352	277	340 (54.0%)	288 (45.7%)	50.2	68.1	UKPDBB
Lynch	Ireland	740	368	372	340 (45.9%)	400 (54.0%)	50.5	70.7	UKPDBB
Lin	Taiwan	320	160	160	160 (50%)	160 (50%)	62.0	70.8	UKPDBB
Facheris	Italy	181	114	67	86 (47.5%)	95 (52.4%)	63.0	NA	UKPDBB
Maraganore	USA	1024	801	223	600 (58.5%)	361 (35.3%)	59	74.7	Bower
Mellick	Australia	2024	1012	1012	1042 (51.4%)	981 (48.4%)	59	72.2	Bower
Morrison	England	1120	766	354	606 (54.1%)	514 (45.8%)	66.1	NA	UKPDBB
Mok	China	436	260	176	264 (60.5%)	170 (38.9%)	NA	63.5	UKPDBB
Aasly	Norway	1278	656	622	721 (56.4%)	557 (43.5%)	58.8	72.9	UKPDBB
Wirdefeldt	Sweden	299	83	216	147 (49.1%)	152 (50.8%)	65.8	71.4	Gelb
Van Broeckhoven	Belgium	1010	501	509	500 (49.5%)	509 (50.3%)	60.5	66.3	Pals/Gelb
Rogaeva	Canada	560	387	173	303 (54.1%)	257 (45.8%)	49.7	64.2	UKPDBB
Tan	Singapore	391	194	197	244 (62.4%)	147 (37.5%)	59.7	54.0	UKPDBB
Hattori	Japan	121	121	0	62 (51.2%)	59 (48.7%)	NA	NA	UKPDBB
Gasser/Sharma	Germany	760	760	0	479 (63.3%)	281 (36.9%)	58.9	NA	UKPDBB
Toda	Japan	306	227	79	161 (52.6%)	145 (47.3%)	57.8	65.1	UKPDBB
Total		15 383	8870	6513			59.5	67.6	

AAO, Age at onset; NA: Not applicable.

variant is located in the C-terminal region of the VPS35 protein pointing that subtle structural changes might influence the disease pathogenesis.[3]

The spectrum of proteins involved in PD aetiology has grown considerably. This includes proteins that are related to mitochondrial quality control (Parkin, PINK1 and DJ1), proteins involved in protein aggregation (SNCA) (Synuclein, MAPT) Microtubule associated protein Tau), and proteins which are involved in sorting and degradation within endocytic and autophagy pathways ((VDAC) Voltage dependent anion channel, (GBA) Glucocerebrosidase gene, VPS35).[16 17] So far, very little is known about the specific role of VPS35 in PD, except that it is hypothesised that it is involved in cargo recognition as part of a retrograde complex recycling membrane proteins from endosomes to the trans-Golgi network.[3 4] Indeed, in vitro and in vivo studies strongly implicate the role of VPS35

Table 2 Clinical description of carriers of non-synonymous variants of vacuolar protein sorting 35 gene

Id	Ethnicity	Rare variant	Age at onset	Clinical signs	Bradykinesia	Rigidity	Tremor	Postural instability	L-dopa responsive	Non-motor symptoms	Family history
P-1	Caucasian	p.Asp620Asn	59	Classical PD	+	+	+	+	+	Negative	Negative
P-2	Caucasian	p.Gly51Ser	NA	Control	–	–	–	–	–	Negative	Negative
P-3	Caucasian	p.Gly51Ser	NAV	Classical PD	+	+	+	+	+	Dementia, visual hallucinations	Negative
P-4	Caucasian	p.Gly51Ser	55	Classical PD	+	+	+	+	+	Negative	Negative
P-5	Caucasian	p.Gly51Ser	49	Classical PD	+	+	+	+	+	Negative	Negative
P-6	Caucasian	p.Asp620Asn	37	Classical PD	+	+	+	+	+	Negative	Positive
P-7	Caucasian	p.Asp620Asn	59	Classical PD	+	+	+	+	+	Negative	Positive
P-8	Caucasian	p.Asp620Asn	55	Classical PD	+	+	+	+	+	Negative	Positive
P-9	Caucasian	p.Asp620Asn	66	Classical PD	+	+	+	+	+	Negative	Positive
P-10	Caucasian	p.Leu774Met	41	Classical PD	+	+	+	+	+	Negative	Positive
P-11	Caucasian	p.Leu774Met	65	Classical PD	+	+	+	+	+	Negative	Positive
P-12	Caucasian	p.Leu774Met	65	Classical PD	+	+	+	+	+	Disturbance of gait and balance	Positive
P-13	Caucasian	p.Leu774Met	NA	Control	–	–	–	–	NA	-	Negative
P-14	Caucasian	p.Leu774Met	44	Classical PD	+	+	+ Rest 1st sx	+	+	Autonomic dysfunction	Positive
P-15	Asian	p.Asp620Asn,p. Leu774Met	52	Classical PD	+	+	+	+	+	Negative	Negative
P-16	Asian	p.Gly51Ser		Control	–	–	–	–	–	–	Negative
P-17	Asian	p.Leu774Met	75	Classical PD	+	+	+	+	+	Negative	Negative
P-18	Asian	p.Asp620Asn	43	Classical PD	+	+	+	+	+	Mild cognitive impairment	Positive

NA, not applicable; NAV, not available; PD, Parkinson disease; +positive; –negative.

gene in neurodegeneration. For example, reduced levels of VPS35 have been found in affected brain regions of Alzheimer disease (AD) patients[18] and loss of VPS35 function has been shown to increase the levels of amyloid β and cause synaptic impairment in a mouse model of AD. Furthermore, variants in another member of the VPS family and substrate of retromer complex, SORL1, have been implicated in AD.[19]

In this study, we have focused only on non-synonymous variants identified by Zimprich and colleagues.[3] Of note, we confirmed the pathogenic relevance of the p.Asp620Asn variant which was identified by both studies for familial cases and in sporadic PD. Recently, published studies also identified p.Asp620Asn mutations in PD,[6 20] thus providing support to the role of p.Asp620Asn in PD. In our study, clinically, the symptomatic carriers showed a broad spectrum of clinical phenotypes ranging from typical PD to (DLB) Dementia with Lewy body, so longitudinal evaluation of carriers at risk will provide unique information on the natural course of the disease caused by VPS35. Even though our data support the role of p.Asp620Asn variant in PD, given the fact that the frequency in diverse population is far below <1%, it is likely to be a rare cause of PD worldwide. Nevertheless, sequencing of families is encouraged for identifying additional missense variants which may provide mechanistic insight into the causes of PD.

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